

ANALYSIS OF SOME TEMPERATURE EFFECTS ON DROSOPHILA PUPAE

ROGER MILKMAN AND BERTIL HILLE

*Department of Zoology, Syracuse University, Syracuse, New York, The Rockefeller University,
New York, New York, and Marine Biological Laboratory, Woods Hole, Massachusetts*

Day-old *Drosophila* pupae respond to high temperature treatment in a variety of ways. Three classes of response have been studied (Milkman, 1962, 1963). Adaptive responses follow relatively gentle treatments; morphogenetic changes result from moderate treatments; and more severe treatments lead to death. Even when observations are restricted to genetically uniform *D. melanogaster* pupae, raised under standard conditions at 23° C. for 25 hours after puparium formation, the qualitative diversity of response is impressive (Milkman, 1962). Considering the wings alone, reduction in size, anterior crossvein defects, posterior crossvein defects, holes, approximation of the third and fourth longitudinal veins, and adventitious appearance of vein material are each stereotyped responses to certain treatments; and these effects do not appear in order, depending simply upon duration of treatment; rather, there are specific and clear-cut qualitative associations between treatment temperature and response. Death, too, appears in unexpected ways; for example, 4¼–4½ hours at 37.5° is lethal to males. The same durations at 38.0° are not. Moreover, the kinetics of the responses are often unusual: the morphological response to 36.5° reaches a peak at 200 minutes, then drops.

This welter of phenomena is forbidding indeed, but there is a body of responses to a large category of treatments that is accessible to analysis. These responses have already been ordered tentatively; the present paper is largely to confirm the order, to add substance and detail, and to lay the foundation for a quantitative scheme capable of defining the kinetics of the several underlying events for a wide range of simple and complex temperature treatments. The purpose of such a scheme is two-fold: to serve as a clear model of complex temperature responses and to summarize the great number of observations that have been made in the present study.

We shall be concerned with the production of posterior crossvein defects at temperatures above 39.0°, with death, and with the protection against these responses conferred by exposure for certain durations to temperatures above 27°.

To review the evidence and conclusions presented to date, we can consider the results of treatments of various duration at 40.5°. It will be useful to refer to Figure 1. Very short treatments (5–10 seconds) followed by an interval at 23° increase resistance to killing and to the production of crossvein defects by subsequent exposures to high temperatures. This resistance is *transient*, wearing off in a couple of hours. We speak of two transitions, A → B at 40.5° and B → C (transient protection) at 23°. By comparing the durations at temperatures between 28° and 40.5° which produce effects similar to those of 5 or 10 seconds at

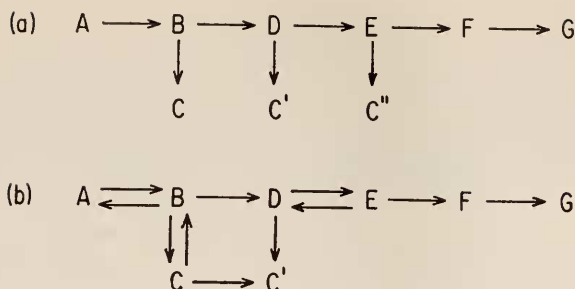


FIGURE 1. The total scheme as originally presented (A) and in its present form (B). The arrows represent transitions considered possible between the hypothetical states represented by letters.

40.5°, we assign $A \rightarrow B$ a one-degree temperature coefficient (Q_1) of 1.5. $B \rightarrow C$ is independent of temperature in the range observed.

Similarly, durations of 20–120 seconds at 40.5° followed by an interval at room temperature produce a *lasting* increase in resistance. The lasting nature of the protection is quantitatively inconsistent with the simple notion that the $A \rightarrow B \rightarrow C$ process has proceeded to a greater extent; therefore $B \rightarrow D$ at 40.5° and $D \rightarrow C'$ (lasting protection) at room temperature are postulated. This time a Q_1 of about 1.8 is obtained for $B \rightarrow D$. $D \rightarrow C'$ is again temperature-independent ($Q_1 = 1.0$).

A longer initial duration at 40.5° (on the order of 5 minutes) enables a subsequent treatment at temperatures down to 32° to produce posterior crossvein defects. This is related to a $D \rightarrow E$ transition. An interval at 23° still increases resistance to subsequent treatment. An $E \rightarrow C''$ step originally suggested has an alternative for which there is now evidence: a reverse change, $E \rightarrow D$, followed by $D \rightarrow C'$.

After a longer period (20 minutes) at 40.5°, an interval at 23° no longer confers protection, and so we have $E \rightarrow F$. But the appearance of defects begins only some time after this transition is essentially complete, so $F \rightarrow G$. Thus it is only in the transition $F \rightarrow G$, in the present scheme, that heat treatments result in crossvein defects. The total scheme as originally presented (Milkman, 1963) is shown in Figure 1a. In the light of additional evidence, a modified form seems more probable (Fig. 1b). The individual transitions here are not thought of as between successive, discrete physiological states, but rather as overlapping conversion processes at a level underlying the physiological states. As a working hypothesis, supported but unprovable by kinetics alone, it is supposed that the transitions are among several tertiary states of a single protein. The letters in the scheme can be taken to stand for these tertiary states, and it is possible to think of several existing at one particular time. In any pupa, the number of molecules in each tertiary state would determine the observable physiological state.

Naturally, such a set of conversions will under many circumstances of time and temperature present a complex picture (see Figure 2 in Hille & Milkman, 1966). The careers of various states will be hard to extricate. So in designing the experiments to be described, the general purpose has been to seek out the circumstances when one particular process stands out, and to study it; for with diverse temperature coefficients the various processes relate differently to one another at various tem-

peratures—being prominent in some and obscure in others. How the appropriate conditions were found will be described for each experiment.

In this paper, it will be impossible to ignore certain assumptions relating the transitions: that they connect the branched sequence of formal compartments (A, B, C, etc.); that they have first order kinetics; and that temperature affects only the rate of individual processes. These assumptions will, of course, have some significance in what is said. But the direct description and test of the set of relationships proposed is made in another paper (Hille and Milkman, 1966). For now, explicit discussion will be limited essentially to the individual steps and their kinetics.

MATERIALS AND METHODS

An inbred Oregon R strain of *Drosophila melanogaster* was used for all the experiments. Flies were raised in an incubator at 23°–25° until puparium formation, then in a Precision water bath at 23° until treatment. After treatment the pupae were returned to the 23° water bath for at least 12 hours and allowed to complete their development in the incubator at 23°–25°. The temperature was thus very closely controlled during the period when it is known to influence posterior crossvein formation and controlled fairly well throughout the life-cycle.

Water baths were controlled with Micro-set thermoregulators and monitored from time to time with thermometers calibrated to 0.01°. A YSI telethermometer was used with a thermistor (time constant 0.8 sec.) to confirm the thermal uniformity of the various regions of the bath. Warmup time (room temperature to within a degree of 40.5°) for pupae in teabags was shown to be less than 4 seconds. Even this technique was not sensitive enough, so more recently, unambiguous histochemical studies of the thermal inactivation of succinic dehydrogenase were used to show that larvae in teabags reach within 1° of 64° in less than 2 seconds.

Treatments totalling less than 40 minutes were generally made in teabags; longer ones employed vials, or else pupae were treated first in teabags and then transferred to vials for completion of the treatment. Crossvein defects were rated from 0 (normal) to 12 (both posterior crossveins completely absent) as previously described (Milkman, 1963).

RESULTS

Early protection

We shall speak of C and C' as protected states because they are off the effective pathway from A to the damaging state G. The distinction between C and C', and therefore between B and D, rests on the transience of protection due to C, as opposed to the lasting nature of protection due to C'. It has been shown that very short exposures to 40.5° at times between 21 and 24 hours, followed by an interval at 23°, protect against crossvein defect production by subsequent exposure to 40.5°. The effect of interval length is distinguishable from that of the pupal age at which the protective treatment was given (Milkman, 1963). As C protection wears off, the comparison in terms of protection conferred between short-interval and long-interval treatments permits the resolution of C formation from C' formation. The lasting protection found after longer intervals requires a longer initial treatment at 40.5°. In order to reconcile a long interval with a test treatment at the time

of peak sensitivity, 25 hours, the pretreatments were originally given at 21 hours (Milkman, 1963). More recently, in anticipation of making a unifying model for the events at 25 hours, these experiments were repeated in detail with pretreatments at 24 hours and test treatments at $25\frac{1}{2}$ hours. The results were essentially identical. Therefore, although the kinetics of at least one later step in the overall sequence must be different at the two ages (see the age-response curve for defect production [Milkman, 1962]), the kinetics through D formation are similar.

TABLE I

Onset and decline of quick protectability at 40.5° ; y seconds at 40.5° + x minutes at 23° + 18 minutes at 41.5° data expressed in rating units

y		x								
		$\frac{1}{2}$	1	2	3	4	5	6	8	10
0	M	5.7	5.8	5.4	5.6	4.3	3.8	5.1	3.4	1.8
	F	6.5	6.7	6.9	6.5	6.2	6.6	6.2	7.2	3.2
5	M	5.4	5.5	4.8	4.5	4.1	1.7	2.0	0.3	0.0
	F	6.1	6.4	6.1	5.9	6.7	5.0	3.0	1.4	0.3
10	M	4.5	5.1	4.1	3.9	2.4	2.1	1.0	0.1	0.1
	F	5.2	6.3	6.4	5.8	5.2	4.6	1.9	0.9	0.3
15	M	4.8	4.5	4.6	2.1	2.5	2.1	0.5	0.1	0.3
	F	5.8	5.9	5.9	4.3	4.4	2.9	2.0	0.6	0.7
20	M	4.9	5.1	5.0	4.1	2.4	1.6	0.4	0.1	0.0
	F	6.1	5.6	6.1	5.3	4.9	3.6	1.5	0.2	0.6
30	M	4.8	5.7	4.5	4.7	2.7	1.5	0.3	0.2	0.1
	F	5.3	5.1	4.7	4.9	4.4	3.5	1.8	0.4	0.1
60	M	4.6	3.8	2.9	4.6	1.9	1.2	0.1	0.3	0.1
	F	5.1	4.7	4.5	4.9	3.9	3.0	2.5	1.4	0.1
120	M	3.6	5.4	3.5	4.2	2.7	2.3	1.2	0.6	0.2
	F	4.4	5.5	4.2	4.6	3.5	4.1	3.1	1.7	0.8
180	M	5.0	5.6	5.6	5.2	5.4	3.3	2.0	0.8	0.3
	F	4.7	4.9	6.9	6.2	6.4	3.9	3.9	3.2	1.4
240	M	5.8	6.4	6.5	6.2	5.5	5.5	4.4	3.4	1.6
	F	7.0	7.1	7.8	7.9	7.0	6.0	4.6	4.4	1.7
300	M	6.5	8.0	6.0	6.5	6.8	6.8	7.2	5.4	2.4
	F	6.0	7.5	8.0	6.8	8.0	7.7	7.0	7.3	3.1
360	M	—	—	—	—	—	—	—	5.2	4.8
	F	—	—	—	—	—	—	—	8.0	dead
480	M	—	—	—	—	—	—	—	—	7.0
	F	—	—	—	—	—	—	—	—	dead

Although the onset at 40.5° of protectability (presence of a protectable state) is rapid, its decline is slower. Moreover we may distinguish the relatively fast conversion of B and D to protected states from the much slower conversion of E. The data in Table I show the rapid onset and slower decline of what we may call "quick protectability." Reading across, the ratings fall as the interval (x) increases. As the duration of the first treatment (y) increases, changes in protectability are best seen by reading down the right hand columns. For example, with 6-minute intervals protectability is great after only 10 seconds at 40.5° , while

several minutes at 40.5° are required to progress beyond this stage. Here the protection against crossvein defects is much more dependent on interval length than on pretreatment duration over a large range. Were it not for the previously established distinctions between transient and lasting protection and between the Q_1 's of 1.5 and 1.8, these data would suggest simply a rapid buildup and slow conversion of a rapidly protectable state. As things stand, however, it appears more likely that two states, B and D, exist which are indistinguishable in this experiment.

The disappearance of D coincides with the formation of E, and the decline of rapid protectability thus coincides with the onset of increased morphogenetic sensitivity to temperatures below 39° . Two types of attempt have accordingly been made to measure specifically the $D \rightarrow E$ transition; one measures the decline in protectability, and the other measures the increase in response to a selected temperature below 39° .

We shall first describe the type of experiment which measures the decline in protectability. As we shall see, in one form it is used to measure the disappearance of D, and in another form it is used to measure the disappearance of E. This type of experiment, called a split series, involves a treatment of fixed length split into two parts by an interval. The variable is the placement of the interval. In either case a total exposure to 40.5° (or a nearby temperature) is chosen which ordinarily produces severe crossvein defects but little reduction in survival. An interval at a lower temperature is interposed at each of various times in the treatment. To the extent that the flies are protectable by the interval, the final defect production will be reduced. Figure 2 presents such data. Ten minutes at 37.5° are sufficient to convert a large amount of any D present to C'. B could also be converted to C, but in fact the B is essentially gone after even the shortest first treatment used here. The 10-minute interval at 37.5° will not convert a significant amount of E to a protected state. Thus this split series with a 10-minute interval at 37.5° is used to chronicle the disappearance of D.

An interval at 23° will convert B, D and E to C or C'. In principle, such an interval can be used to monitor the amount of E, which of course remains substantial for some time after the disappearance of D. Figure 3 contains the results of such experiments. Notice by comparing these data to those of Figure 2 that 23° protection is indeed still seen after first treatments too long to be mitigated by a 37.5° interval. To maximize this 23° protection, one would like to use a 60- to 90-minute interval, since conversion of E to C' takes a long time. But intervals of this length force either the early or the late part of the high temperature treatment out of the maximally sensitive period, and so 30 minutes is used as the best compromise.

Although temperatures other than 40.5° can be used for the treatment, the number of events taking place makes it impossible to calculate temperature coefficients for single transitions by this method. Note that teabags are used whenever possible to minimize warmup time; even when the total elapsed time from beginning to end of treatment is too great for a teabag to be used exclusively, it can be used for 20 minutes at 40.5° , after which no protection at 23° is observed, and the pupae are then transferred to vials. The transfer period has no mitigating effect at this time.

To return to D decay, the data in Figure 2 show that, at 40.5°, D is essentially gone after 8 minutes. Further displacement of the interval results in no great increase in ratings, indicating no further loss in 37.5° protectability. At higher temperatures, particularly 42.5°, the overall process of crossvein defect production (appearance of G in amounts exceeding a threshold) takes place so rapidly that one cannot monitor the total disappearance of D. At these temperatures a substantial amount of D is still present when enough G has been formed to cause serious crossvein defects, and even death. This is consistent with the observation that the overall process leading from A to G has a Q_1 of 2.3, while D formation has a Q_1 of 1.8 and the conversion of D to E, though not accessible to exact measurement, seems also to have a Q_1 well below 2. Thus the conversion of a certain amount

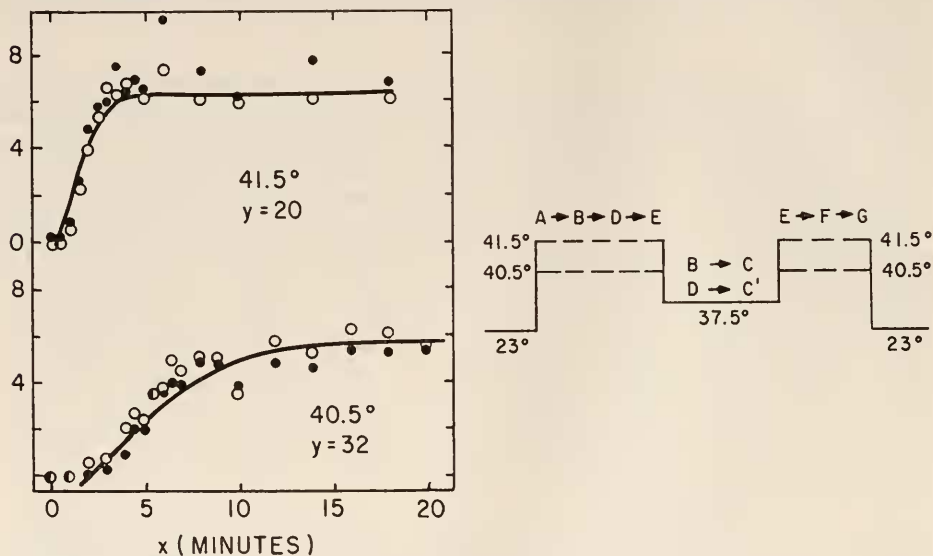


FIGURE 2. Disappearance of D as measured by split series experiment with interval at 37.5° C. Interval begins x minutes after start of treatment; y = total duration (minutes) at 40.5° or 41.5°. As D disappears, the protective effect of 37.5° interval declines; rating thus rises as onset of interval becomes later. Open circles, females; filled circles, males. Temperature sequence and reactions associated with each step diagrammed at right. Dashed horizontal line, variable duration; solid horizontal line, fixed duration.

of D into E will be followed by its subsequent conversion to G before all the D disappears. And this amount of G is enough to cause extreme responses. Why the latter part of the sequence—why indeed the overall sequence—has such a high temperature coefficient will be explained in terms of the D branch point to be discussed shortly.

The other measure of the D → E transition monitors E formation rather than D disappearance; in other words, it follows the increase in sensitivity to lower temperatures rather than the loss of protectability. This kind of experiment involves a variable first treatment at 40.5° (or nearby) and a constant second treatment at a temperature below 39°. The rationale here is that with successively

longer first treatments, the second treatment should become more and more effective in causing crossvein defects because in terms of our scheme, as more E is made, more F can be made at the lower temperature. Thus the response would rise sharply with increased first treatment length, reflecting E formation primarily. After the E is all made (essentially), a further increase in first treatment duration would increase the total response much less, merely in relation to the lengthening of the total treatment; we would see, in a simple case, something approaching a broken

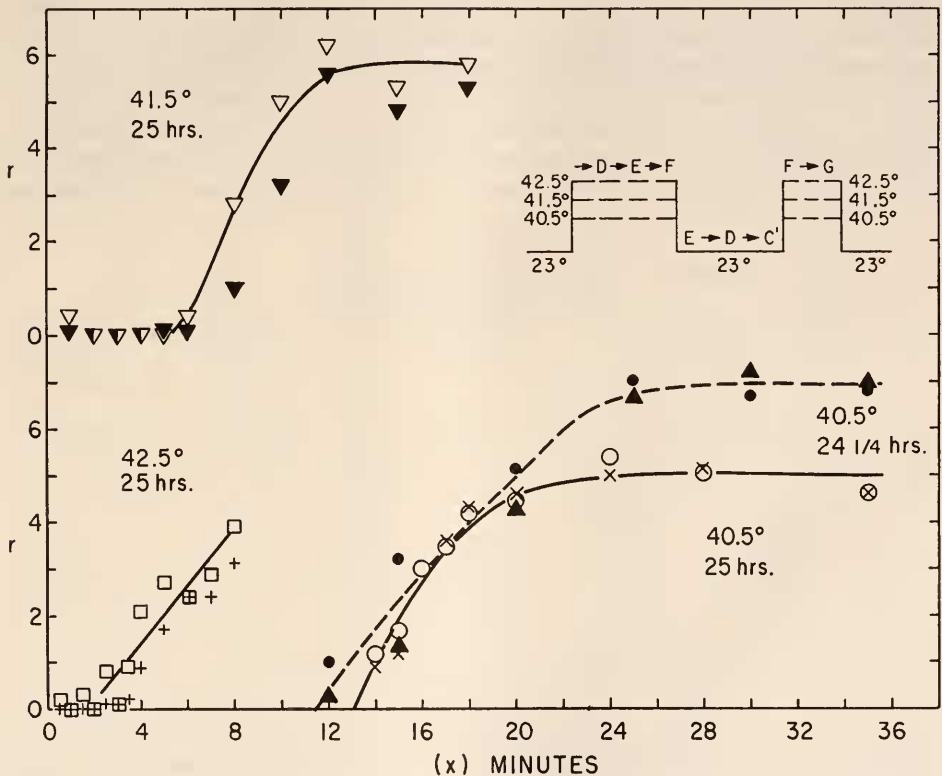


FIGURE 3. Disappearance of E as measured by split series experiment with interval at 23° C. Interval begins x minutes after start of treatment. Total duration at 40.5° = 35 minutes; at 41.5°, 18 minutes; at 42.5°, 8 minutes. As E disappears, the protective effect of 23° interval declines; rating thus rises as onset of interval becomes later. Females: 40.5°, circles; 41.5° and 42.5°, open triangles and squares. Males: remaining symbols.

curve. In practice, as might be predicted (from the apparent overlap of the various transitions, and from the necessarily unequal total treatment durations), the results do not form such a simple pattern. Accordingly, the split series experiments are the experiments of choice. Nevertheless, the broken curve experiments have been of some value (Milkman, 1963), and the data obtained fit the predictions made with our analog computer (Hille and Milkman, 1966).

The conversion of E to F, too, has a measurable course at 40.5° but not at

42.5°. The dose which produces death limits the length of time over which one can follow D decay or E decay. Once more, it appears possible (at 42.5°) to produce a lethal amount of the terminal form before *completing* the transitions in the middle of the sequence. This illustrates the overlaps which we must consider.

The protection of E has been studied in terms of a response *vs.* interval length experiment. Because of the length of time required to protect E, such an experiment runs the risk of extending beyond the sensitive peak period. However, short intervals produce enough information to permit some conclusions. A simple hypothesis is an $E \rightarrow C''$ transition, as was previously proposed (Milkman, 1963). One would expect a great amount of protection with a short interval and a progressive reduction in added protection as interval length increased. An alternative notion of comparable simplicity would involve the reversal of the $D \rightarrow E$ transition, with E going back to D, which will then go to C' , as we have already described. Here one might expect (depending on the actual quantitative characteristics) a lag first, then protection as one increased the interval. The kinetics of protection

TABLE II
Kinetics of E protection; 40.5° (10 min.) + 34.0° (x min.) + 40.5° (y min.).
Data expressed in rating units

x	Males			Females		
	y					
	25	30	37	25	30	37
0	8.0	d	d	8.9	d	d
10	8.6	10.3	(10.0)	8.3	9.7	d
20	7.2	9.2	(9.8)	7.0	9.1	d
30	5.4	8.2	8.8	5.8	7.4	8.8
40	4.4	6.4	8.0	4.6	6.3	7.7
50	3.5	4.1	5.3	4.3	5.4	5.1
60	1.8	3.2	3.7	3.0	3.8	4.0

would not be first order, even if each process were a monomolecular event. The experimental results are shown in Table II. They favor the indirect $E \rightarrow D \rightarrow C'$ path, since there is a lag of 10 or 20 minutes, after which protection increases linearly with interval. Accordingly, we presently believe that protection of E involves its conversion back to D and then to C' .

After about 20 minutes at 40.5°, essentially all the A has gone either to F or to the protected states. No further protection is possible. This is true, not only for 25-hour pupae but for younger and older ones as well. The evidence for this comes from a comparison between two kinds of age-response curves. The first relates responses to 35 minutes at 40.5° with respect to pupal age. The second deals with split treatments: 20 minutes at 40.5° are administered at a given age, and the rest at 25 hours, the age of peak sensitivity (Milkman, unpublished). These curves are essentially parallel (the split treatment curve shows greater response, due to the contribution of the treatment at peak sensitivity), but treatments much shorter than 20 minutes at any age, followed by room temperature, confer protection.

Since the 20-minute first treatment is the significant variable when administered at or before 25 hours, and since the $F \rightarrow G$ transition is not important until later, it appears that one or more of the early steps is age-dependent, resulting in a variable proportion of A going to a protected state in the first 20 minutes.

After 25 hours, however, the latter part of the treatment is the important variable, indicating either that $F \rightarrow G$ slows down with increasing age, or that the effect of G production on posterior crossvein formation decreases with time. The cross-

TABLE III

Results of treatment completion at lower temperatures; first treatment: 25 minutes at 40.5°

Second treatment		r	
Temperature (°C.)	Duration (min.)	♂	♀
32.5	250	10.4	9.9
	200	8.8	8.5
	150	6.2	6.5
	100	4.3	6.3
	50	2.7	4.4
	40	1.6	3.2
31.5	240	8.8	8.7
	120	3.8	5.6
30.5	240	7.9	7.1
	150	4.4	6.3
	120	2.5	5.1
	90	2.5	4.5
	60	1.8	4.1
29.5	240	7.9	7.9
28.5	240	4.5	6.7
27.5	240	4.0	5.0
26.5	240	2.6	4.5
24.5	240	0.5	1.5
22.5	240	0.1	1.6
18.5	240	0.0	1.1

vein is being laid down at this time (Mohler and Swedberg, 1964; Waddington, 1940).

The last step in the sequence, $F \rightarrow G$, proceeds much more slowly than the others, and it has been the easiest to study with respect to its temperature coefficient. Merely by treating the pupae for 25 minutes at 40.5°, one reaches a stage where essentially only this reaction is going on. Previous data (Milkman, 1963) on the amount of subsequent time at various temperatures needed to produce a certain

average defect have indicated a Q_1 of 1.4–1.5 down to 33.5°. Further experiments have extended this relationship down to 26.5°, below which temperature the durations required would be excessive. The new data are given in Table III and illustrated, together with the previous data, in Figure 4. These results, incidentally, point up the importance of controlling temperature after treatments.

Although the temperature coefficients are accessible to study, the actual rate of the process is not easy to describe in terms other than average amount of defect produced per unit time. If $F \rightarrow G$ is a first order process, it is very slow indeed, since the relationship between dose and response has a linear appearance. Only

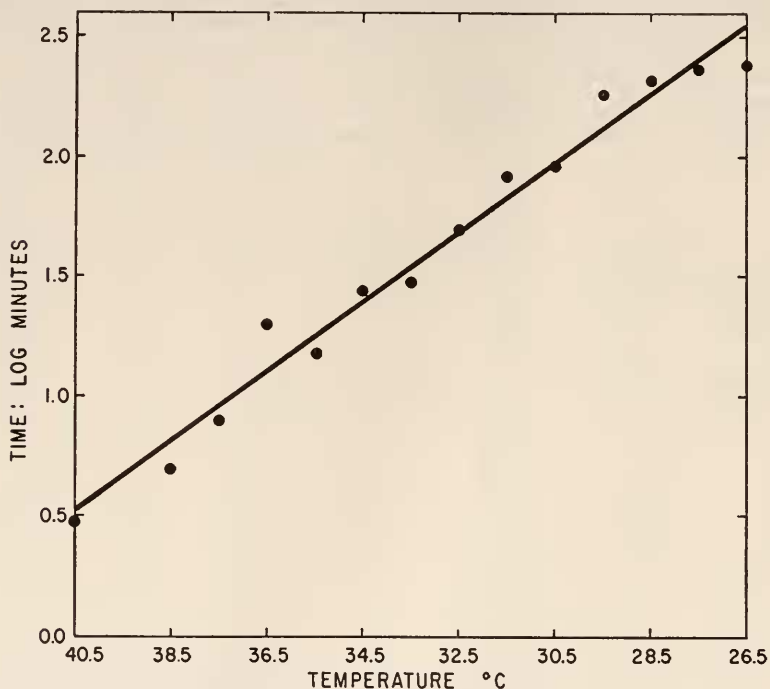


FIGURE 4. Determination of the temperature coefficient of the $F \rightarrow G$ transition. Ordinate: log duration at a given temperature which, when added to a 25-minute exposure to 40.5° C., produces a criterion response (sum of male and female ratings = 7). Visually plotted line corresponds to $Q_1 \cong 1.4$.

the early part of a first order reaction appears linear. This, in turn, would imply that very little loss of the hypothetical functional protein would lead to morphological defects and that very little further loss would lead to death. This is not unreasonable in a living organism, particularly since we have sought out this developmental process on the basis of its sensitivity. There is no present basis for further speculation, however.

The measurements of overall temperature coefficients at higher temperatures, difficult in vials because of the significant warmup period, have been extended through the use of teabags. The durations at temperatures from 40.0° to 42.5°

required to produce a criterion response (sum of male and female ratings = 6.0) have been compared and are plotted in Figure 5. These data yield an overall Q_1 of 2.0, somewhat lower than the value previously obtained (2.3) but still higher than the Q_1 for any individual step.

The paradox thus remains that the overall Q_1 appears to be between 2.0 and 2.3, while the individual steps appear to have Q_1 's no higher than 1.8, with the Q_1 of the slowest reaction, $F \rightarrow G$, being 1.4–1.5. It was proposed (Milkman, 1963) that the overall Q_1 represented a product of two kinds of temperature functions. One, the temperature coefficient of the rate, is familiar, but the other is less obvious. It is the proportion of a form proceeding to one of two possible subsequent forms. We illustrate with D because it appears in practice to be the only relevant case *at*

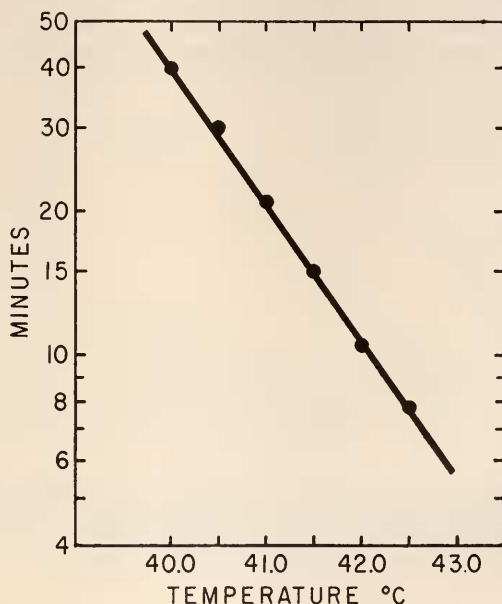


FIGURE 5. Determination of the overall temperature coefficient. Ordinate: duration at a given temperature which produces a criterion response (sum of male and female ratings = 6). Visually plotted line corresponds to $Q_1 = 2.0$.

the temperatures we are considering. Since D can go to C' and also to E, and since $D \rightarrow C'$ has a temperature coefficient of 1 (is temperature-independent) and $D \rightarrow E$ has a temperature coefficient in the neighborhood of 1.7, we can imagine a low temperature at which all the D goes to C', a high temperature at which all the D goes to E, and an intermediate range over which the proportion of D going to E is a sensitive function of temperature. In a first order system, the rate of formation of F is $k_{EF} [E]$, and since k_{EF} and $[E]$ are both temperature-dependent, the rate must depend on the product of these temperature-dependent functions. Similarly the rate of formation of F must have a two-factor temperature dependence even if we treat the pupae at a single temperature from beginning to end; and dG/dt must share this dual dependence. Thus the overall temperature coefficient,

$Q_1 = 2.0$ – 2.3 , could be factored into the product of 1.4 – 1.5 ($Q_{1\text{FG}}$, $F \rightarrow G$ being the slowest reaction in sequence) times 1.3 – 1.6 , which we attribute to the temperature dependence of the proportion of D going to E.

Experimental support for this hypothesis comes from examining the response to 39.5° of pupae which have largely reached the F stage at one of a number of experimental temperatures. Clearly the rate constant of $F \rightarrow G$ at 39.5° , k_{FG} , is fixed. But if the total amount of E made, and therefore the total amount of F made, is temperature-dependent, then the dose-response relationship at 39.5° will depend upon the temperature at which the early part of the treatment was given. The results are illustrated in Figure 6, supporting qualitatively the hypothesis that the nature of the Q_1 of 2.0 – 2.3 is compound. The slopes of the dose-response curves show the relationship between rate of G synthesis at 39.5° and the temperature at which E was made. The increase in slope is about 1.3 per degree, within the range 1.3 – 1.6 predicted crudely here, and in good quantitative agreement with

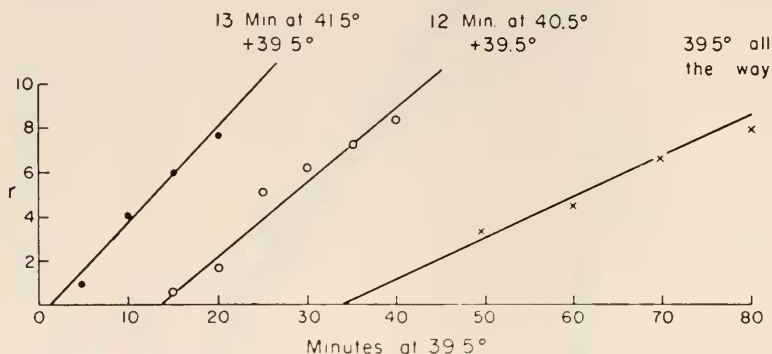


FIGURE 6. Dependence of dose-response relationship at 39.5° upon temperature of first part of treatment. Examples selected for purposes of effective display. Data from additional experiments at 41.5° and 40.5° where first treatments have different durations show similar slopes. Lines drawn from computer calculations (Hille and Milkman, 1966).

our computer predictions, which can and must take a number of additional factors into account (Hille and Milkman, 1966).

It is noteworthy that, no matter what the assortment of treatments within the present range (and specifically excluding 4 hours at 37.5° reported previously [Milkman, 1963] and described as not capable of inclusion in the present scheme), an average defect rating of 9 or more is always accompanied by high mortality and 11 is virtually unattainable, apparently because of death. It is possible to protect against crossvein defects so that death comes first; but it is not possible to produce extreme crossvein defects and yet retain good survival. Thus it seems that the hypothetical protein involved in crossvein formation is also vital—presumably elsewhere in the organism—and there it has a slightly greater reserve which can be destroyed before measurable effects appear. When death occurs in pupae protected against crossvein defects, it requires a longer treatment than usual and is presumably due to an event which ordinarily is preceded by death due to the destruction of the crossvein protein.

One can calculate first order rate constants for each of the transitions, on the basis of the relationship $k = 0.69/t_{1/2}$, where $t_{1/2}$ is the time for the reaction to proceed halfway. One might expect tertiary structure transitions in proteins to have first order kinetics, since many denaturation processes do. The rate constants have thus been calculated for all the processes that reach completion (essentially). For $F \rightarrow G$, which comes nowhere near completion, not even enough curvature can be seen in its time curve to permit a calculation of a rate constant. This fact does set an upper limit, though, which enables us, rather speculatively, to set the rate constant in the range of 0.01. Table IV lists all the first order rate constants.

An heuristic aspect of setting rate constants in this way—based on the individual processes—is that we can try to see whether in concert they are consistent with a

TABLE IV
*First order rate constants for the individual steps**

Transition	Rate constant (min. ⁻¹) at 40.5°C.
A $\rightarrow$ B	10
B $\rightarrow$ C	0.15
B $\rightarrow$ D	4.0
D $\rightarrow$ C'	0.15
D $\rightarrow$ E	0.10
E $\rightarrow$ F	0.15
F $\rightarrow$ G	0.01**

* Estimates are given for the forward steps only; for complete list of constants, see Hille and Milkman, 1966.

** Very rough estimate; see text.

wide variety of results at various single temperatures and combinations of temperatures. Such an analysis is to be found in another paper (Hille and Milkman, 1966).

DISCUSSION

The work described in this paper is largely confirmatory and raises no new issues (*cf.* Milkman, 1963). The minor changes in the overall scheme are the dropping of one tertiary state (C'') and the introduction of a reverse arrow between D and E. It appears from the body of information now accumulated that the various transitions are valid, both in themselves and in relation to the others. Moreover the practical limit seems to have been reached in terms of the experimental dissection of steps in the overall process. There are certain temperatures at which some transitions are prominent; the $E \rightarrow F$ transition, however, is not resolvable over a broad enough range to calculate a temperature coefficient.

The notion that the transitions are related in a sequence undercuts the calculation of an original amount of A, relative to a threshold and relative to crossvein defect rating units, by the simple extrapolation of G formation. Similarly, the branch ratio at D cannot be considered in isolation, as one factor of a two-factor product, if one is to systematize the several events and their outcome over a broad temperature range. Now that the best estimates obtainable from these

experiments are at hand for the time course of each transition, and since the postulated tertiary structure changes suggest first order kinetics and easily calculated rate constants, it is of theoretical and practical value to place the calculated values in the postulated scheme. By so doing, one can attempt, with the aid of an analog computer, to predict the results of an array of simple and complex treatments that would otherwise be difficult to approach. This is done in another paper (Hille and Milkman, 1966).

Very little conclusive information has been added lately with respect to the mechanism of temperature adaptation and other temperature effects, although such effects are being studied in a number of theatres, with findings that are interesting in themselves and as leads (Ushakov, 1964; Prosser, 1966). Since Northrop's work (1920) on temperature adaptation in *Drosophila*, which was conveyed from one generation to the next *via* the egg, the observations on this animal and others have been intriguing but neither explained nor thoroughly generalized. Mohler's (1965) finding that not all parts of the posterior crossvein respond identically to high temperatures in flies of certain genotypes makes it clear once again that the present scheme holds in a restricted theatre at best.

The idea of tertiary structure change has little direct experimental support. One of us (R. M.) has attempted using both *Drosophila* extracts and pure proteins (bovine serum albumin, hemoglobin) to demonstrate heat-induced deviant tertiary structure states as expressed by altered migration rates in acrylamide gel disc electrophoresis. After numerous failures, recent efforts with larval extracts treated at 55° have been successful (Milkman, in Prosser, 1966). These experiments are still preliminary and we know of no similar work; yet it is logical at present to allow for the transition of some proteins among various stable or meta-stable tertiary structure states, rather than restricting them to a single-structured condition whose only alteration would be denaturation to a disordered state. For further discussion of this subject, see Milkman in Prosser (1966).

Finally, it should be emphasized that the collected evidence presented here strongly supports a single branched sequence, whatever the fundamental mechanism, because of the indissociability of the steps from one another and the impossibility of rearranging them.

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SUMMARY

1. A previously presented scheme unifying a variety of high temperature effects on day-old *Drosophila melanogaster* pupae has been confirmed substantially by a large body of new data. Several minor modifications have been made.

2. New experiments confirm the validity of analyzing heat-induced temperature adaptation over a several-hour period at the end of the first day of pupal development.

3. Several new types of experiments have been described which involve combinations of exposures to more than one temperature and which provide information on the transitions between several intermediate stages in the scheme.

4. A double temperature-dependence is demonstrated in which both the amount of a precursor and the rate of its conversion vary with temperature in a particular range. In this range, the temperature coefficient of crossvein defect induction is the product of two components. This property inheres in branched pathways.

5. Rate constants and temperature coefficients have been calculated for the individual steps on the basis of data from specific experiments.

6. The scheme incorporates the information from a large array of detailed experiments and is capable of generalization over a broad temperature range.

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